Bioinorganic Chemistry

The Copper Chaperone NosL Forms a Heterometal Site for Cu Delivery to Nitrous Oxide Reductase

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Abstract: *The final step of denitrification is the reduction of nitrous oxide (N_2O) to N_2, mediated by Cu-dependent nitrous oxide reductase (N_2OR). Its metal centers, Cu_A and Cu_Z, are assembled through sequential provision of twelve Cu^I ions by a metallochaperone that forms part of a nos gene cluster encoding the enzyme and its accessory factors. The chaperone is the nosL gene product, an 18 kDa lipoprotein predicted to reside in the outer membrane of Gram-negative bacteria. In order to better understand the assembly of N_2OR, we have produced NosL from Shewanella denitrificans and determined the structure of the metal-loaded chaperone by X-ray crystallography. The protein assembled a heterodinuclear metal site consisting of Zn^{II} and Cu^I, as evidenced by anomalous X-ray scattering. While only Cu^I is delivered to the enzyme, the stabilizing presence of Zn^{II} is essential for the functionality and structural integrity of the chaperone.*

Introduction

Nitrous oxide reductase (N_2OR) is the terminal oxidoreductase in microbial denitrification, where nitrogen derived from biomass is recycled back into the atmosphere.[1] It mediates the reduction of nitrous oxide (N_2O) to dinitrogen gas (N_2) and water.[1] Although highly exergonic at $\Delta G^{0'} = -339.5 \text{ kJ mol}^{-1}$, this 2-electron reduction is challenging due to the chemical inertness of nitrous oxide that manifests in an activation energy barrier of 250 kJ mol^{-1}. N_2OR is a two-domain protein that utilizes two multinuclear copper centers (Figure 1 A).[2] In a C-terminal cupredoxin-type domain the enzyme binds the binuclear copper center Cu_A that is very similar to the site of the same name in cytochrome *c* oxidase.[3] Its oxidized state is a mixed-valent [$Cu^{1.5+}$:$Cu^{1.5+}$], and it is liganded by two histidine residues, two cysteines, one methionine and a backbone carbonyl.[4] Cu_A accepts and transfers a single electron, with a midpoint potential of

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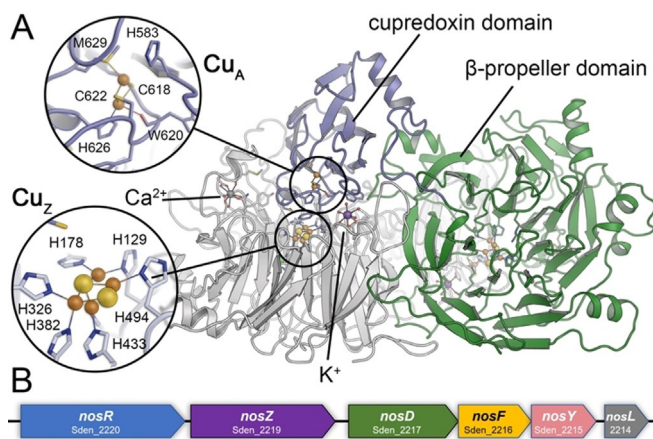https://doi.org/10.1002/anie.202106348.

Figure 1. Architecture of N_2O reductase and its metal sites, Cu_A and Cu_Z. **A)** N_2OR is a 130 kDa homodimer consisting of an N-terminal β-propeller and a C-terminal cupredoxin domain. The tight head-to-tail dimer juxtaposes the cupredoxin domain with the binuclear Cu_A of one monomer with the tetranuclear Cu_Z in the hub of the propeller domain of the other monomer, creating a composite active site for binding, activation and reduction of the inert greenhouse gas N_2O. Monomer A of the enzyme is shown in white, monomer B with the β-propeller domain in green and the cupredoxin domain in blue. **B)** Structure of the *nos* gene cluster of *Shewanella denitrificans* encoding for nitrous oxide reductase and the corresponding electron transfer and maturation machinery. The *nosR* gene encodes a putative quinol oxidase required for electron transfer to N_2OR, while *nosZ* is the structural gene for the enzyme itself. *NosD, nosF* and *nosY* form an ABC-transporter complex that is essential for Cu_Z maturation, and *nosL* is the copper chaperone analyzed in the present work.

$+260 \text{ mV}$ for the enzyme from *Pseudomonas stutzeri*.[5] The second metal site, Cu_Z, is a unique [4Cu:2S] cluster coordinated by 7 histidine residues in the hub of a seven-bladed β-propeller domain.[2,6] Cu_Z binds and activates N_2O for reduction during catalysis (Figure 1 A).[2] In the 130 kDa periplasmic metalloprotein N_2OR, two chains form a tight head-to-tail homodimer that closely aligns the Cu_A site of one monomer to the Cu_Z cluster of the other, creating the complex active site of the enzyme at and around the dimer interface (Figure 1 A).[7] Although apo-N_2OR is exported from the cytoplasm in a folded state via the Tat system, the assembly of both metal sites occurs exclusively in the periplasm. It requires an intricate machinery of accessory transporters, reductases and chaperones around the cytoplasmic membrane and in the periplasmic space that afford a comprehensive assembly line, preventing undesired side reactions and assuring the specific delivery of all required components to their appropriate physiological targets.[1b]

In the denitrifying Gammaproteobacterium *Shewanella denitrificans* N$_2$OR is encoded by the *nosZ* gene that forms part of a *nos* gene cluster with the canonical structure *nosRZDFYL* (Figure 1B).[1a] NosR, a polytopic transmembrane iron-sulfur flavoprotein, is the physiological electron donor for N$_2$O reduction.[8] The open reading frames *nosDFY*, located downstream of *nosZ*, the structural gene for N$_2$OR, encode a multi-subunit ABC-type transporter that is strictly required for the formation of Cu$_Z$.[1,9] Herein, NosF is the cytoplasmic ATPase, NosY forms the integral membrane domain of the transporter with six transmembrane helices and NosD was suggested to be an associated carrier protein located in the periplasm.[1b,10] *NosL*, the subsequent gene in the cluster, is present in most genomes of denitrifying organisms, including those in which the relative order of the *nosDFYL* genes is not conserved, suggesting an important role in N$_2$OR assembly.[1a,9b] NosL is a lipoprotein of 18 kDa that was proposed to be anchored to the inner leaflet of the outer membrane of the Gram-negative bacterium via a conserved N-terminal cysteine that gets lipidated after translocation through the inner membrane.[11] As NosD is presumably anchored in the cytoplasmic membrane via the ABC transporter NosFY, this raises the question how copper-bound to NosL crosses the periplasm to reach NosD.

NosL from *Achromobacter cycloclastes* specifically and stochiometrically binds Cu^I, supporting its involvement in copper delivery to N$_2$OR as a metallochaperone.[12] The NMR solution structure of an apo form of NosL from *A. cycloclastes* heterologously produced in *E. coli* revealed a close structural homology to the organomercury lyase MerB and implicated one of the conserved residues, Met109, in metal binding. However, the entire N-terminal region of apo-NosL was disordered in the NMR structure, and while this was in line with NosL acting in copper delivery during N$_2$OR maturation,[11a] little was learned about the site and mode of metal binding. In *P. stutzeri*, the Tn5-mediated disruption of the C-terminus of NosL had no effect on N$_2$OR synthesis.[13] Furthermore, the expression of the *nosZDFY* genes from *P. stutzeri* in *Pseudomonas putida*, a related but non-denitrifying organism, was sufficient for the production of catalytically competent N$_2$OR, confirming that NosL is not indispensable for Cu$_Z$ or Cu$_A$ biosynthesis or—more likely—that there is a functional backup system present in these particular hosts.[9b,11a] A further caveat may be that copper was added to the growth media in these studies, so that a dedicated chaperone may still be essential under conditions of metal limitation.

The complex role of NosL was further highlighted in recent studies on different denitrifiers. NosL of *Paracoccus denitrificans*, for instance, binds a single Cu^I per protein with attomolar affinity and is specifically required for Cu$_Z$ center assembly in N$_2$OR.[14] More recently, we reported the recombinant production of active *P. stutzeri* holo-N$_2$OR in *Escherichia coli*, where we found the ABC transporter NosFY and the accessory NosD protein to be required for the formation of the catalytic Cu$_Z$ site, but not Cu$_A$.[15] In this study, however, the enzyme from *Marinobacter hydrocarbonoclasticus* could not be matured at all, independent of *nosL* coexpression, while the Cu$_A$ site of *S. denitrificans* was

correctly assembled only if *S. denitrificans* NosL was present.[15] We therefore chose *S. denitrificans* as a source organism for the present study, so as to assure that the Cu$_A$ site in NosZ can only be formed if a functional NosL protein is coproduced.

Results and Discussion

To facilitate the isolation and crystallization of NosL we generated a soluble version with a cleavable leader peptide and a C-terminal StrepTag(II) that lacked the lipidation site, residue Cys38, of the wild type protein. *S. denitrificans* NosL (*Sd*NosL) was produced recombinantly in *E. coli* and was isolated as a monomer (Figure S1). The pure protein showed no UV/vis absorption features beyond 280 nm, but although the medium was not further supplemented with metals, an ICP-MS analysis revealed 0.56 Zn^{2+} ions per NosL monomer, but only 0.02 Cu. From here, the holoprotein could be reconstituted in vitro by providing Cu^I under anoxic conditions.

Cu-reconstituted *Sd*NosL crystallized in the cubic space group $F4_132$ with one monomer per asymmetric unit, allowing for the determination of its structure to 1.85 Å resolution by the single-wavelength anomalous dispersion method (Figure 2A, Table S2). In good agreement with the apo-NosL
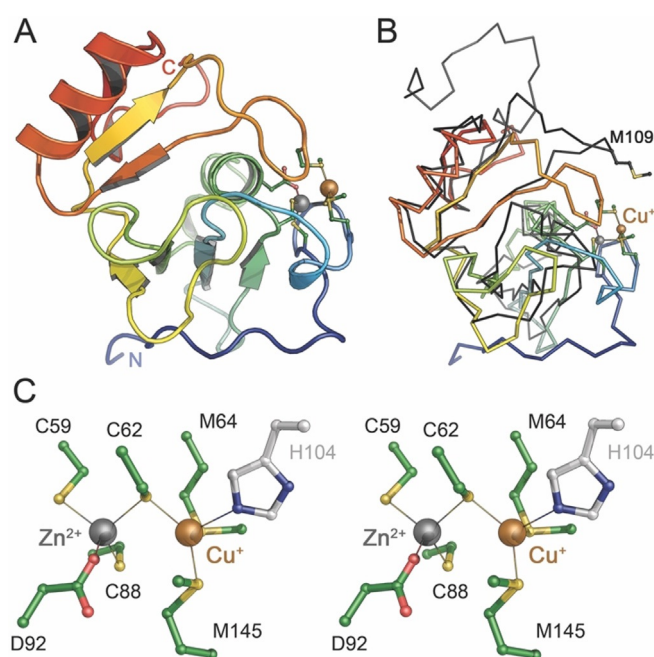

Figure 2. Structure of *Sd*NosL. A) Cartoon representation of holo-*Sd*NosL, colored from blue at the N-terminus to red at the C-terminus. B) Superposition of *Sd*NosL with one model from an NMR ensemble of apo-NosL from *A. cycloclastes*. In the demetallated protein the N-terminus is highly flexible until residue 73, encompassing most of the metal-binding region. Methionine residue M109, corresponding to M145 of *Sd*NosL, is situated in a loop that shows substantial flexibility within the ensemble. C) The heterobimetallic metal site of the protein is formed near the N-terminus and contains one Zn^{2+} and one Cu$^+$ cation. Copper is additionally coordinated by a histidine residue from a neighboring monomer in the crystal packing.

structure of *A. cycloclastes* that aligned to our model with a root-mean-squared deviation of 2.3 Å for all atoms, the overall fold of *Sd*NosL consists of two structurally similar, independent domains of four and three antiparallel β-sheets and a mixed β/α topology with a characteristic ββαβ motif in each domain (Figure 2 B).[11a] In contrast to the NMR solution structure, however, the N-terminal part of *Sd*NosL was well-ordered, likely due to the coordination of two metal ions close to the surface of the protein.

In the dinuclear metal site of NosL, both metal ions adopted a distorted tetrahedral geometry (Figure 2 C). With five sulfur-containing residues in a total of seven amino acids ligands, the site appeared quite unlike any other known biological metal center, and the identification of the nature of the metal cations was of obvious importance (Figure 3 A). We collected three diffraction data sets around the X-ray absorption K-edges of Cu and Zn (Figure 3 B), at energies of (i) 8958 eV, (ii) 8998 eV and (iii) 9678 eV. From these, anomalous double difference ($\Delta\Delta_{ano}$) electron density maps were calculated for Zn ((iii)−(ii)) and Cu ((ii)−(i)). These maps allowed for the unambiguous identification of the more buried metal as zinc (Figure 3 C), while the one at the surface of NosL was identified as copper (Figure 3 D). Importantly, this analysis also revealed that the population of both sides is

unique, with each species fully restricted to its designated position.

Despite the presence of molecular oxygen during crystallization the crystals remained colorless (Figure S1), indicating a reduced state of the copper atom and a resistance to oxidation upon exposure to air. The Cu-Zn distance was 3.4 Å, and most of the coordinating ligands belonged to the N-terminal domain that was disordered in the apo-NosL structure: Cys59 is a ligand to Zn^{II}, Met64 coordinates Cu^{I} and Cys62 is μ_2-bridging both metals (Figure 2 C). Cys88 and Asp92, also part of the N-terminal region, coordinate zinc, and the hard carboxylate of Asp92 as a ligand strongly disfavors the coordination of a second, soft Cu^+ ion at this position. The environment of copper, however, consists exclusively of soft sulfur ligands that will favor Cu^+ over the harder Cu^{2+}, and in addition the more flexible methionine sidechains of residues M64 and M145 will facilitate uptake and release of copper, while zinc is held tightly in place.

The only ligand originating from the C-terminal part of NosL is Met145, and the C-terminal loop where it is located seems to undergo a major conformational rearrangement upon Cu binding, as indicated by a superposition of the crystal structure of *S. denitrificans* holo-NosL with an ensemble of the 20 lowest energy NMR structures of *A. cycloclastes* apo-NosL (Figure 2 B).[11a] Only in one of these 20 states the loop in question pointed towards the Zn,Cu binding site, revealing a possible transition state on the way from the apo- to the holo-form, where Zn^{II} is already bound and the repositioning of Met109 readies the system for Cu^{I} capture (Figure S2). In addition, the heterobimetallic site in holo-NosL was coordinated by a seventh ligand, but this His104 derived from another NosL monomer within the crystal, leading to the formation of a homotrimer *in crystallo* (Figure 2 C, Figure S3). This histidine ligand shows a limited degree of conservation among NosL proteins of other N_2O-reducing organisms (Figure S4), while the other Cu-binding residues, Cys62, Met64 and Met145, are strictly conserved among fifteen analyzed NosL homologs. Note that not all of these form part of a canonical *nos* cluster, indicating the protein to be a more widely used type of metallochaperone. It cannot be ruled out that the observed coordination of His104 to the Cu^{I} ion is only induced by packing interactions within the crystal lattice, and we therefore conducted a geometry optimization of the heterobimetallic binding site of NosL in the absence of the external ligand. Interestingly, this led to an inversion of the tetrahedral geometry of the Cu ion and its retraction into the chaperone, creating a more symmetric dinuclear site with both Cys62 and Cys88 serving as bridging ligands (Figure 4 A). Although only a model, this structure suggests a chemically reasonable metal-loaded resting state for a monomeric NosL protein. The known orthologs of NosL show substantial variability in metal coordination. For instance, the two cysteine residues coordinating the Zn ion are not fully conserved, so that zinc may not be present in all NosL-type Cu chaperones. Five of the analyzed organisms retain Cys59, Cys88 and Asp(/Glu)92. Among these exclusively Zn-binding ligands, Cys59 is the most highly conserved, while the positions of Cys88 and Asp92 are swapped in the
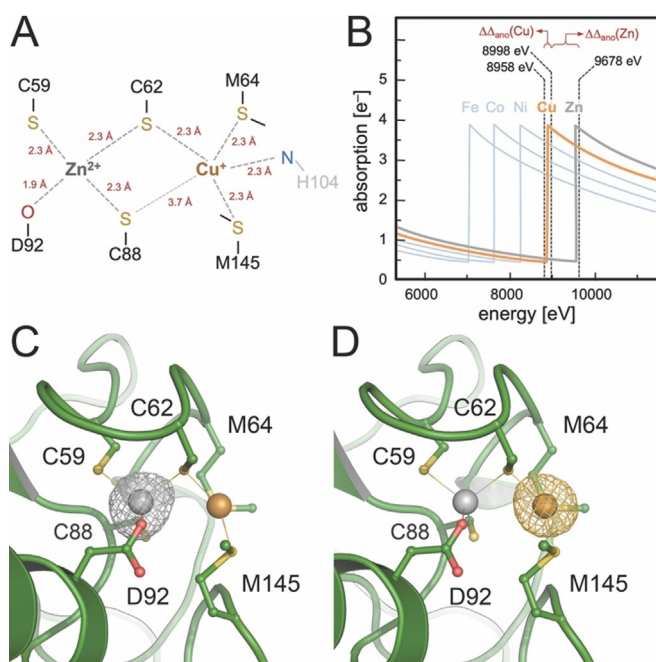

Figure 3. The heterodinuclear metal site of *Sd*NosL. **A)** Architecture of the Zn,Cu-site in holo-NosL. Bond distances are given in Ångströms. Most ligands originate from the N-terminal region and domain 1. **B)** Plot of the X-ray absorption K-edges for the last five 3rd row transition metals. Dashed lines indicate the wavelengths where data sets were collected to obtain the $\Delta\Delta_{ano}$ maps depicted in C) and D). **C)** The metal site with a $\Delta\Delta_{ano}$ map for Zn, obtained as the difference of anomalous scattering information collected at 9678 eV and 8998 eV. **D)** The metal site with an analogous $\Delta\Delta_{ano}$ map for Cu, as a difference of anomalous data collected at 8998 eV and 8958 eV. Both maps are contoured at the 10σ level and show that no mixture of the metals occurs.
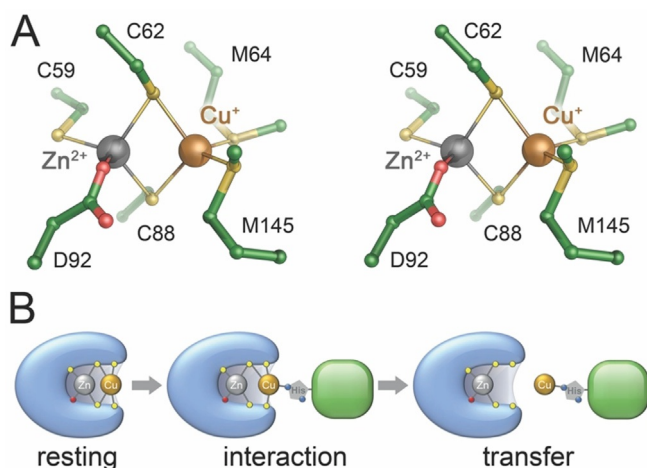
Figure 4. A putative resting state for holo-NosL. **A)** After removal of the external His ligand, a geometry-optimized metal site in NosL features two μ_2-bridging cysteines in a symmetric arrangement. **B)** A model for Cu trafficking from NosL to an interaction partner (NosD and/or NosZ). An exposed histidine on the partner protein first activates Cu^I from the resting state to the position observed in the crystal structure of holo-NosL. The ion can then leave NosL bound to the partner protein, while Zn^{II} is retained in the chaperone to stabilize the N-terminal region of the protein.

NosL proteins from three of the analyzed N_2O-reducers that belong to clade II (Figure S4).

For zinc-containing proteins, the first coordination sphere of the metal ion typically reflects its function as either a structural, catalytic or regulatory moiety.[16] Accordingly, the cysteine-rich coordination sphere of Zn^{II} and its buried position in holo-NosL suggest a predominantly structural role, possibly by organizing and stabilizing the N-terminus of the protein, providing a stable yet flexible environment for the reversible uptake of Cu^I mediated by the strictly conserved Cys62 and Met64 located in this region (Figure S4). Hereby, zinc can stabilize the protein structure and prevent intramolecular disulfide conformation, as was suggested for the Zn-dependent sulfurtransferase SufU from *Bacillus subtilis* that exhibits the same 3Cys:1Asp Zn^{II} coordination as found for holo-NosL.[17] An additional role may be in preventing the unspecific binding of zinc to N_2OR or other proteins by scavenging the metal ions. As recombinant *S. denitrificans* N_2OR produced in *E. coli* only contained copper in its Cu_A site when *Sd*NosL was coproduced,[15] we proceeded to generate the Zn^{II} ligand variants C59G[L], C88A[L] and D92A[L], as well as a C88D/D92C[L] swap to mimic the situation in clade II N_2OR, in order to assess the relevance of Zn^{II} for the copper-donating role of NosL. Indeed, the coproduction of each of these mutants with *S. denitrificans* N_2OR resulted in a decreased Cu content of the enzyme as determined by ICP-MS (Figure S5J). In addition, the correct assembly of the Cu_A site of N_2OR was impaired, as only very little Cu_A signal was observed after oxidation with ferricyanide (Figure S5A–D), indicating that both residues—and hence the ability to bind Zn^{II}—are essential for copper trafficking by NosL. Individual replacement of the conserved, sulfur-containing Cu ligands of NosL (C62A[L], M64A[L],

M145A[L]) consistently resulted in apo-N_2OR when coproduced and isolated (Figure S5E–G). This confirmed the importance of the three strictly conserved ligands to copper and their role in copper transfer from NosL to N_2OR. In contrast, the swap of C88[L] and D92[L] did not yield a functional NosL, suggesting that the properties of clade II NosL proteins also must differ in other aspects (Figure S5I). The coproduction of H104A[L], on the other hand, resulted in a correctly assembled Cu_A site in N_2OR, albeit with a further decreased copper content (Figure S5 H,J), indicating that His104, which is responsible for the trimer formation observed in the crystals of *S. denitrificans* NosL (see Supplementary Discussion), is not essential for the uptake and transfer of copper. This is underlined by the lack of conservation of His 104 among NosL orthologs. It may, however, replace a physiological histidine ligand supplied by the interaction partner of NosL, likely apo-N_2OR (NosZ) and/or the NosDFY system that is required for Cu_Z formation. In this way, the coordination of Cu by His104 (Figure 2 A), together with the modeled structure of a putative metal-loaded state (Figure 4 A) outline the first two steps of the mobilization of Cu from the chaperone when the metal ion is eventually transferred to the apo-enzyme (Figure 4 B).

Conclusion

Our analysis of the metal-replete state of the copper chaperone NosL revealed the unexpected integration of a Cu^I ion into a heterobimetallic binding site together with Zn^{II} that presumably plays a predominantly structural role. In its apo form, The N-terminus of NosL is disordered, and the association of a zinc ion that is bound with high affinity is required to pre-form a reversible binding site for copper that must be of lower affinity to facilitate the transition of the metal from and to its interaction partners. According to its character, Cu^I is bound via soft sulfur ligands, including cysteines that may be prone to forming disulfide bonds upon oxidation. Avoiding this may well be a secondary function of the coordinated zinc. The serendipitous interaction of the heterobimetallic site with a non-conserved histidine residue from a neighboring monomer within the crystal lattice is highly suggestive of a possible pathway for copper delivery to an interaction partner, and we propose that in the absence of a suitable ligand the Zn,Cu site is likely to attain a more symmetric conformation that represents a metal-loaded resting state. It remains to be determined whether NosL will functionally interact with NosZ or NosDFY or even both. As NosL is predicted to be a lipoprotein residing in the inner leaflet of the outer membrane, an interaction with the soluble NosZ protein is well conceivable, while NosD seems to be permanently anchored to the cytoplasmic membrane. It remains to be clarified if and how an interaction of NosL and NosD can be achieved or whether the assembly steps for Cu_A (via NosL) and Cu_Z (via NosD) are spatially separated.

Conflict of Interest

The authors declare no conflict of interest.

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